Objective—microRNA-155 (miR155) plays a critical role in immunity and macrophage inflammation. We aim to investigate the role of miR155 in atherogenesis.

Approach and Results—Quantitative real-time polymerase chain reaction showed that miR155 was expressed in mouse and human atherosclerotic lesions. miR155 expression in macrophages was correlated positively with proinflammatory cytokine expression. Lentivirus-mediated overexpression of miR155 in macrophages enhanced their inflammatory response to lipopolysaccharide through targeting SOCS-1 and impaired cholesterol efflux from acetylated low-density lipoprotein–loaded macrophages, whereas deficiency of miR155 blunted macrophage inflammatory responses and enhanced cholesterol efflux possibly via enhancing lipid loading–induced macrophage autophagy. We next examined the atherogenesis in apolipoprotein E–deficient (apoE−/−) and miR155−/−/apoE−/− (double knockout) mice fed a Western diet. Compared with apoE−/− mice, the double knockout mice developed less atherosclerosis lesion in aortic root, with reduced neutral lipid content and macrophages. Flow cytometric analysis showed that there were increased number of regulatory T cells and reduced numbers of Th17 cells and CD11b+Ly6CCh+ cells in the spleen of double knockout mice. Peritoneal macrophages from the double knockout mice had significantly reduced proinflammatory cytokine expression and secretion both in the absence and presence of lipopolysaccharide stimulation. To determine whether miR155 in leukocytes contributes to atherosclerosis, we performed bone marrow transplantation study. Deficiency of miR155 in bone marrow–derived cells suppressed atherogenesis in apoE−/− mice, demonstrating that hematopoietic cell–derived miR155 plays a critical role.

Conclusions—miR155 deficiency attenuates atherogenesis in apoE−/− mice by reducing inflammatory responses of macrophages, enhancing macrophage cholesterol efflux and resulting in an antitherapeutic leukocyte profile. Targeting miR155 may be a promising strategy to halt atherogenesis.

Key Words: atherosclerosis ■ inflammation ■ macrophages

Atherosclerosis is both a lipid storage disease and a chronic inflammatory process. Although lipid accumulation in the intimal space and local inflammation are closely linked in atherogenesis, macrophages are pivotal players in the process through both maintaining vessel wall lipid homeostasis and orchestrating inflammatory responses. Numerous molecules take part in the pathological transformation of macrophages in atherogenesis, and the complex signaling network underlying macrophage inflammation in the context of atherogenesis is far from clear.

MicroRNAs (miRs) are short noncoding RNA molecules that regulate gene expression post-transcriptionally through base pairing with mRNAs, resulting in either translational repression or mRNA degradation. They are estimated to regulate up to a third of all human genes and play critical roles in many physiological or pathological processes, including cell differentiation, immunity development, and cancer transformation. Therefore, miRs are emerging as new targets for the diagnosis and therapy of human diseases, including dyslipidemia and atherosclerosis. MicroRNA-155 (miR155) is encoded in the B-cell integration cluster (Bic) gene and is prominently expressed in many hematopoietic cell types; it has been demonstrated to be oncogenic and play a crucial role in immune response regulation. In macrophages, a microarray analysis found that miR155 was among the few miRs that were substantially upregulated by Toll-like receptor (TLR) ligands. Although the functional relevance of macrophage
miR155 expression is unclear, studies indicated that miR155 may enhance inflammatory response by stimulating the release of inflammatory mediators by targeting several negative feedback molecules including SOCS-1 and SHIP-1. However, it may also attenuate inflammatory response under other circumstances. Given the critical role of macrophage inflammation in atherogenesis, it is imperative to define the effect of miR155 expression and regulation on atherogenesis. Recently, 2 studies showed opposite results on the effects of bone marrow–derived cells reduced atherogenesis in apoE−/− mice. In 1 study, Donners et al23 reported that bone marrow miR155 deficiency increased atherogenesis in LDLR−/− mice fed a high-fat diet by generating a more proatherogenic immune cell profile and a more proinflammatory monoocyte/macrophage phenotype. In the other study, Nazari-Jahantigh et al24 showed that miR155 promoted atherogenesis in a partial carotid ligation apolipoprotein E–deficient (apoE−/−) mouse model by repressing Bcl6 in macrophages, thus enhancing vascular inflammation. In the current study, we first show that miR155 expression was increased in mouse and human aorta atherosclerotic lesions and that miR155 expression was positively correlated with proinflammatory cytokine expression under various conditions. Furthermore, increased miR155 expression conferred macrophages a proatherogenic phenotype, including enhanced inflammatory responses to lipopolysaccharide (LPS) and impaired cholesterol efflux on cholesterol loading, whereas miR155 deficiency rendered macrophage less inflammatory. Moreover, we compared the atherosclerosis development in apoE−/− and apoE−/−/miR155−/− mice and showed that miR155 deficiency in apoE−/− mice attenuated atherogenesis by reducing macrophage inflammation. This result was confirmed by bone marrow transplantation study showing that deficiency of miR155 in bone marrow–derived cells reduced atherogenesis in apoE−/− mice.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

miR155 Is Upregulated in Mouse and Human Aortic Atherosclerotic Lesions

We first examined miR155 expression in mouse aorta atherosclerotic lesions by quantitative real-time polymerase chain reaction (qRT-PCR). miR155 expression was determined in aorta segments from 4-month-old wild-type (WT) C57BL/6 mice and 3- to 7-month-old apoE−/−/LDLR−/− mice. The aortas of the apoE−/−/LDLR−/− mice were dissected into atherosclerotic and nonlesion (normal) segments under microscope. Total RNAs (including miRs) were extracted from the aorta segments for qRT-PCR analysis of miR155 and tumor necrosis factor (TNF) α expression. Figure IA in the online-only Data Supplement shows that the atherosclerotic segments of aortas from apoE−/−/LDLR−/− mice expressed higher levels of miR155 and TNFα compared with the nonatherosclerotic aorta segments of the same apoE−/−/LDLR−/− mice, as well as aortas from WT mice. We further examined miR155 expression in human aorta atherosclerotic lesions by qRT-PCR. Lesional tissue and the surrounding normal tissue from human aortas were dissected, and total RNA was extracted for measurement of human miR155 and TNFα expression. Figure IB in the online-only Data Supplement shows that compared with surrounding normal aortic tissue, human aortic atherosclerotic lesion had 2-fold increase in miR155 expression, along with a 7-fold increase in TNFα expression.

miR155 Expression Is Upregulated in Macrophages by TLR4 Activation and Is Correlated With Inflammatory Cytokines

It is known that a TLR4 ligand, LPS, stimulates miR155 expression in macrophages.25 We first examined whether a more physiologically relevant atherogenic TLR4 ligand, minimally oxidized low-density lipoprotein (mmLDL), induces miR155 expression in macrophages. LDL was minimally oxidized by incubation with copper sulfate (10 μmol/L; 3 hours) to generate mmLDL. The mmLDL was used to stimulate thioglycollate-elicited peritoneal macrophages from WT C57Bl/6 mice, apoE−/−/LDLR−/− mice, and TLR4−/− mice. Cells were also stimulated with LPS as a positive control. The macrophages were incubated for 6 hours with serum-free DMEM, with the addition of either mmLDL (100 μg/mL) or LPS (100 ng/mL). qPCR was performed to quantify miR155 expression. Figure IIA in the online-only Data Supplement shows that both mmLDL and LPS significantly increased macrophage miR155 expression in a TLR4-dependent manner, and the responses were enhanced significantly in macrophages from apoE−/−/LDLR−/− mice than in those from WT C57Bl/6 mice. We further measured miR155 expression levels in macrophages from mice of different genotypes, including WT C57Bl/6, LDLR−/−, apoE−/−, apoE−/−/LDLR−/−, and TLR4−/−, and subjected to various treatments, including DEMEM control, mmLDL or LPS stimulation, or acetylated LDL loading. Concurrently, we also measured the expression levels of inflammatory cytokines in these macrophages. In Figure IIB in the online-only Data Supplement, we plotted the correlation of miR155 levels and TNFα or interleukin-6 (IL-6) mRNA levels in the macrophages, showing a clear positive correlation between miR155 expression levels and TNFα or IL-6 levels.
vector for miR155 overexpression. A 419-bp DNA fragment containing the mouse pre-miR155 stem-loop was amplified by PCR from the C57BL/6 mouse macrophage cDNA and subcloned into the bicistronic lentiviral vector PWPI to generate the PWPI-miR155-GFP construct. This bic DNA fragment is similar to the fragment that Costinean et al used for the generation of the B-lymphocyte–specific miR155 transgenic mouse model. The expression of miR155 and GFP is under the control of the universal promoter EF-1α (Figure IIIA in the online-only Data Supplement). The transduction efficiency for primary macrophages is >90% when a MOI of 30 is used as demonstrated by flow cytometric analysis of GFP expression (data not shown). The overexpression of mature mouse miR155 was confirmed in PWPI-miR155-GFP lentivirus–transduced mouse peritoneal macrophages by qPCR (increased ≤30-fold compared with PWPI-GFP lentivirus transduced cells; Figure IIIB in the online-only Data Supplement). We transduced peritoneal macrophages from WT C57Bl/6 mice with PWPI-miR155-GFP or control PWPI-GFP lentiviruses and examined inflammatory cytokine expression. Interestingly, we found that miR155 overexpression alone did not increase TNFα expression in macrophages. However, when stimulated with LPS, the PWPI-miR155-GFP lentivirus–transduced macrophages displayed a 3-fold increase in TNFα expression compared with the control group (P=0.022; Figure 1A).

To investigate the molecular mechanisms through which miR155 enhances macrophage inflammatory responses to pro-inflammatory stimuli, protein levels of SOCS-1 and SHIP-1 were detected in miR155-overexpressing macrophages. It has been reported that SOCS-1 and SHIP-1, 2 negative regulators of the TLR4-mediated inflammatory pathway, are miR155 target proteins in monocytes and macrophages. Therefore, we examined whether miR155 overexpression reduced protein levels of SOCS-1 and SHIP-1 in mouse peritoneal macrophages. The transduced macrophages were stimulated with LPS (100 ng/mL) for 0, 6, or 24 hours. Protein levels of SOCS-1 and SHIP-1 were examined by Western blot using β-actin as a loading control. The results showed that mouse miR155 overexpression substantially reduced SOCS-1 levels in mouse peritoneal macrophages, whereas SHIP-1 levels were not changed (Figure 1B and 1C).

Figure 1. MicroRNA-155 (miR155) enhances macrophage inflammatory responses by targeting SOCS-1. A, Mouse macrophages were transduced with PWPI-GFP or PWPI-miR155-GFP lentiviruses. The transduced cells were treated with lipopolysaccharide (LPS; 50 ng/mL) for 6 hours before mRNA was extracted for quantitative polymerase chain reaction (qPCR) measurement of tumor necrosis factor (TNF) α expression (n=3 for each group). B, The transduced cells were treated with LPS (50 ng/mL) for 6 to 24 hours. Cell lysates were used for detection of SOCS-1 and SHIP-1 levels by Western blotting. C, Western blots in 3 experiments were analyzed for the relative protein levels (*P<0.05). D–F, Wild-type (WT) and miR155−/− mouse peritoneal macrophages were treated with LPS (10 ng/mL), Pam3CSK4 (500 ng/mL), FSL-1 (100 ng/mL), poly (I:C; 10 μg/mL), and ODN1668 (1 μmol/L) for 6 hours, respectively. Total RNA was extracted and reverse transcribed into cDNA as template to detect the inflammatory cytokines (D, TNFα; E, interleukin-6 [IL-6]; F, IL-1β) by qPCR (n=3; *P<0.05, **P<0.01).
miR155 Deficiency Attenuates Macrophage Inflammatory Response

To confirm the function of miR155 in macrophage inflammatory responses, we compared the macrophages from WT and miR155−/− mice. We treated WT and miR155−/− mouse macrophages with different TLR ligand, including LPS (TLR4 ligand), Pam3CSK4 (TLR1/2 ligand), Fsl-1 (TLR2/6 ligand), Poly(I:C) (TLR3 ligand), and ODN1668 (TLR9 ligand). We found that miR155 deficiency diminished the expression of TNFα, IL-6, and IL-1β induced by all of above TLR ligands, indicating that miR155 deficiency dampened macrophage inflammatory signaling triggered by multiple TLR activation, including TLR2, TLR4, TLR3, and TLR9 (Figure 1C).

miR155 Expression Affects Macrophage Cholesterol Efflux

It has been well demonstrated that macrophage inflammation and cholesterol homeostasis are intimately connected. The contact point is the PPAR-LXR axis. To examine whether miR155 affects macrophage cholesterol homeostasis, we first measured the cholesterol efflux from the macrophages transduced with PWPI-miR155-GFP lentiviruses. Figure 2A shows that, compared with control PWPI-GFP lentivirus–transduced macrophages, miR155-overexpressing macrophages displayed impaired cholesterol efflux to apolipoprotein A-I (a 10% decrease; P=0.035), but efflux to DMEM (baseline cholesterol diffusion) was not changed. We next used peritoneal macrophages from miR155−/− or WT mice to measure cholesterol efflux. We found that miR155 deficiency significantly increased the cholesterol efflux to apolipoprotein A-I (by 45%; P=0.0002), whereas cholesterol efflux to high-density lipoprotein was not significantly different (Figure 2B).

To examine molecular mechanisms underlying the increased cholesterol efflux in miR155−/− macrophages, we loaded WT and miR155−/− macrophages with acetylated LDL for 48 hours, qPCR was used to quantify the mRNA expression, and Western blot was performed to compare the protein expression of ABCA1 and ABCG1. Surprisingly, we found that ABCA1 and ABCG1 in miR155−/− macrophages were comparable to those in WT macrophages (Figure 2C and 2D), indicating that increased cholesterol efflux in miR155−/− macrophages was not because of increased expression of cholesterol efflux genes, ABCA1 and ABCG1. Recent studies have shown that macrophage autophagy enables cholesterol efflux via presenting free cholesterol to cell membrane.26,27 We thus examined whether miR155 deficiency increases autophagy of macrophages, especially under cholesterol-loading condition. We performed Western blot to measure LC3 protein in macrophages and found that both forms of LC3 (LC3-I and LC3-II) were significantly increased in miR155−/− macrophages, both in unloaded and in acetylated LDL–loaded cells, compared with WT macrophages (Figure 2E and 2F), indicating that miR155 deficiency enhances macrophage autophagy.

miR155 Deficiency Attenuates Atherogenesis in Mice

Because macrophage miR155 deficiency attenuated inflammatory responses and enhanced cholesterol efflux, we next examined whether miR155 deficiency protects mice from developing atherosclerotic lesions. Female apoE−/− and miR155/apoE double knockout (DKO) mice at 8 weeks of age were fed a Western-type diet to raise plasma cholesterol levels and to induce atherosclerosis. After 12 weeks on the Western diet, the mice were euthanized. Body weight, plasma cholesterol, and triglyceride levels, as well as fast protein liquid chromatography lipoprotein profiles at the end point, were comparable between both groups of mice (Figure IV in the online-only Data Supplement). As visualized by...
hematoxylin and eosin staining, mean lesion area was 0.48±0.03 mm² in aortic roots of apoE−/− mice and 0.30±0.04 mm² in DKO mice, reflecting a 37% reduction (Figure 3A). However, atherosclerotic lesion area in en face aortas was not statistically significantly different between apoE−/− and DKO mice (Figure V in the online-only Data Supplement).

We further stained the lesions for neutral lipid with Oil Red O. The results showed that lipid staining area in the lesions of DKO mice (0.31±0.02 mm²) was significantly smaller than that in apoE−/− mice (0.20±0.02 mm²), reduced by 36% (Figure 3B). We stained lesional macrophages using a mouse macrophage specific antibody MOMA-2 and found the DKO mice displayed a ≈48% less macrophage area (0.26±0.03 mm²) in aortic root atherosclerotic lesions compared with apoE−/− mice (0.13±0.02 mm²; Figure 3C). However, collagen content in lesions as determined by Movat’s Pentachrome staining was not significantly different between the 2 groups (Figure VI in the online-only Data Supplement).

miR155 is profoundly involved in immunity, modulating immune cell differentiation and maturation. We thus examined whether miR155 deficiency altered leukocyte populations in our atherosclerotic mouse model using flow cytometry. Our data showed that in DKO mouse spleen, the percentages of T cells (CD3+/CD4+, and CD3+/CD8+) and Th17 cells (CD4+/IL-17+) were decreased and those of B cells (CD19+) and regulatory T cells (CD4+/FoxP3+) were increased compared with those in apoE−/− mouse spleens. Although the percentage of CD11+/Ly6C+ cells was not different in apoE−/− or DKO mouse spleens, the percentage of CD11+/Ly6C(high) subpopulation was significantly reduced in DKO mouse spleens (Figure 4).

At the end point, we obtained peritoneal macrophages from apoE−/− and DKO mice and treated the macrophage with vehicle or LPS (100 ng/mL) for 6 or 24 hours. We found that both in the absence and presence of LPS stimulation DKO macrophages expressed TNFα, IL-6, and IL-1β mRNAs at significantly lower levels than apoE−/− macrophages after 6 hours of stimulation (Figure 5A). ELISA analysis showed that after 24 hours of incubation, DKO macrophage–conditioned medium contained significantly lower levels of TNFα and IL-6 compared with the conditioned medium of apoE−/− macrophages (Figure 5B). These data suggest that miR155 deficiency in lipoprotein-loaded apoE−/− macrophages diminished their inflammatory responses. We also measured TNFα and IL-6 concentrations in mouse plasma and found that the concentration of IL-6 was significantly reduced in DKO mice than in apoE−/− mice; however, the plasma levels of TNFα showed no difference in 2 groups of mice.

Bone Marrow miR155 Deficiency Reduces Atherosclerosis in Mice

To dissect further the role of myeloid cell miR155 expression in atherogenesis, we performed a bone marrow transplantation study. We transplanted bone marrow cells from apoE−/− or DKO mice to lethally irradiated apoE−/− mice of 12 weeks of age. After 4 weeks, mice were fed a Western diet for 16 weeks. As in the study using whole body knockouts, apoE−/− recipient mice with miR155 deficiency in bone marrow–derived cells did not have plasma lipid alteration compared with their miR155-WT chimeric controls (data not shown) but had significantly reduced atherosclerosis in aortic root (Figure 6A–6F). Using laser capture microdissection, we collected atherosclerotic lesions from the aortic root. Figure VII in the online-only Data Supplement shows images of aortic root section before and after the atherosclerotic lesion was removed by laser capture microdissection. qRT-PCR confirmed that in the lesions of the recipients of DKO bone marrow, the mRNA expression of miR155 targets Bcl-6 and SOCS-1 was significantly increased compared with that in recipients of apoE−/− bone marrow (Figure 6G). Proinflammatory chemokine CCL2 expression was decreased in DKO bone marrow recipients; however, TNFα expression showed no difference between apoE−/− bone marrow and DKO bone marrow recipients (Figure 6C).

Discussion

miR155 is a unique miR in that it is coded only by 1 gene, called bic, and is the only product of this gene.28–30 miR155-deficient mice are the first miR knockout mouse model,13,14 providing a unique opportunity to investigate the role of a miR in pathophysiology. miR155 has been demonstrated to play important roles in immunity and inflammation, especially macrophage inflammatory responses, implying that it may also be involved in atherogenesis. Indeed, 2 recent studies have shown that miR155 deficiency in bone marrow cells affected atherosclerosis.
However, the results are contradictory. Although one report showed that Western diet–fed LDLR−/− mice transplanted with miR155-deficient bone marrow developed more atherosclerotic lesions than mice received WT bone marrow, indicating that in this model miR155 is atheroprotective,23 the other showed that bone marrow miR155 deficiency reduced plaque size after partial carotid ligation in apoE−/− mice, suggesting that miR155 is proatherogenic.24 The primary reason for this contradiction was that miR155 deficiency enhanced macrophage inflammation in hypercholesterolemic LDLR−/− mice as shown in the first study23 whereas it attenuated macrophage inflammation in another hypercholesterolemic mouse model (apoE−/− mice) as shown in the second study.24 Our current study provided comprehensive data and demonstrated that whole body or bone marrow miR155 deficiency profoundly reduced macrophage inflammation and atherosclerosis in the aortic root region in Western diet–fed apoE−/− mice. However, atherosclerosis in aortic arch and descending aortic regions was not different between the 2 genotypes. Region-specific effects have been reported in many atherosclerosis studies.31,32 In apoE−/− mice fed a Western diet for 3 months, atherosclerotic lesions are most profound in the aortic root, whereas lesions are relatively scant in the descending region. It is possible that lack of significant difference in the descending region was because of relatively small lesions in this region. However, the definitive mechanism of this region-specific effect remains to be defined.

In agreement with the recently published study,24 we found that miR155 expression was increased in both mouse and human atherosclerotic lesions. We confirmed that minimally oxidized LDL and LPS stimulated macrophage miR155 expression via TLR4-dependent pathway. Moreover, we showed a positive correlation between miR155 expression level and proinflammatory cytokine expression level in macrophages of various genotypes and under varied treatment including cholesterol loading. Furthermore, using both gain-of-function and loss-of-function approaches, we showed that baseline expression of miR155 substantially affected inflammatory responses of macrophages to TLR ligands. In addition, consistent with previous reports, we found that miR155 suppressed the expression of SOCS-1, a negative feedback protein of macrophage inflammation.21,33 This was also confirmed in vivo by laser capture microdissection and qPCR. In atherosclerotic lesions of apoE/ miR155 DKO bone marrow recipient mice, the expression of SOCS-1 and another miR155 target, Bcl6, was significantly increased compared with that in the lesions of apoE−/− bone marrow recipient mice. Our data strongly suggest that in macrophages proinflammatory cytokines and miR155 may reciprocally regulate each other, and thus miR155-targeting strategies may lead to macrophage inflammation suppression. Donners et al23 showed that in normal conditions, miR155 ablation attenuated inflammatory responses in macrophages, whereas in oxidized LDL–loaded macrophage foam cells, miR155 deficiency paradoxically promoted a more proinflammatory phenotype. We, however, found that macrophages from Western diet–fed apoE/miR155 DKO mice produced less proinflammatory cytokines either at baseline or under LPS stimulation compared with the macrophages from Western diet–fed apoE−/− mice. The reason for this discrepancy is unknown, possibly because of an unknown alteration caused in their study by in vitro oxidized LDL loading or in vivo excessive cholesterol overloading in Western diet–fed LDLR−/− mice whose plasma total cholesterol level may reach ≈1500 mg/dL,25 which is exceptionally high (in our study, the plasma cholesterol level of the mice at the end point was 700–750 mg/dL). In addition, the status of apoE may alter significantly the function of miR155 in macrophages. The exact reasons for the difference in the 2 different models warrant further investigation.

Macrophage inflammation and cholesterol accumulation are closely linked together in atherogenesis.34 Imbalance between cholesterol uptake and efflux in the context of hyperlipidemia leads to macrophage foam cell formation, which is an obligatory step of atherogenesis. In this study, we demonstrated for the first time that overexpression of miR155 compromised whereas miR155 deficiency improved cholesterol efflux from...
acetylated LDL–loaded macrophages. Mechanistically, we demonstrated that the enhanced cholesterol efflux by miR155 deficiency in macrophages was not because of increased expression of cholesterol transporters ABCA1 and ABCG1, rather at least partially because of increased autophagy. Autophagy is a cellular process that assists the cell to achieve a homeostasis under stress. Recently, elegant work by Ira Tabas and colleagues has shown that macrophage autophagy plays an antiatherogenic role, and Yves Marcel and colleagues further demonstrated that autophagy was activated in macrophage foam cells and facilitated ABCA1-mediated cholesterol efflux. Previous studies have shown that miR155 knockout impaired the activation of Akt. Inhibition of Akt pathway has been shown to induce autophagy. Moreover, Akt1 inhibits miR155 expression in macrophages, which would constitute a negative feedback loop. However, the detailed mechanism by which miR155 regulates macrophage autophagy and cholesterol efflux warrants further investigation. Consistent with the cholesterol efflux result, the atherosclerotic lesions in DKO mice contained less Oil Red O–stained region and fewer macrophages than those in apoE−/− mice. While we were revising this article, 2 groups reported that miR155 may modulate macrophage autophagy on Mycobacteria infection. One report showed that forced miR155 expression accelerated the autophagic response in macrophages, whereas the other study found that miR155 inhibited interferon-γ–induced autophagy. These results indicate that the role of miR155 in macrophage autophagy is complex and context dependent.

In addition to macrophage functional alteration because of miR155 deficiency, we also found that miR155 deficiency resulted in an overall antiatherogenic spleen leukocyte profile, evidenced by increased regulatory T cell and reduced CD11b+/Ly6Chigh cell percentages. miR155 has been suggested to confer competitive fitness to regulatory T cells; the increased regulatory T cells in miR155−/− mice in our study reflect the possibility that the effects of miR155 on regulatory T cell may be altered by hyperlipidemia. Many studies have shown that regulatory T cells are antiatherogenic, whereas CD11b+/Ly6Chigh cells are proatherogenic. However, our study does not provide direct evidence whether the increased...
miR155 is proinflammatory in macrophages. New studies, including ours, suggested that miR155 expression is proatherogenic. Inhibition of myeloid cell miR155 expression would yield antiatherogenic benefit through suppressing macrophage inflammatory responses, enhancing macrophage cholesterol efflux, and achieving an antiatherogenic circulating leukocyte profile.

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**Disclosures**

None.

**References**

35. Du et al miR155 Deficiency Attenuates Atherosclerosis. 9
MicroRNA-155 Deficiency Results in Deceased Macrophage Inflammation and Attenuated Atherogenesis in Apolipoprotein E–Deficient Mice
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Supplemental materials

**Supplemental Figure I**

**miR155 expression is increased in mouse and human aortic atherosclerotic lesions.**

A. miR155 and TNFα expressions in aorta were analyzed by qRT-PCR. WT: wild type C57Bl/6 mice. E/L-lesion and E/L-normal: aorta segments with atherosclerotic lesions or without lesion (normal) from apoE-/-/LDLR-/— mice, respectively. (*p<0.05, vs WT; **p<0.01, vs WT and E/L-normal). B. Expression levels of miR155 and TNFα were significantly increased in atherosclerotic lesions from human aortas, compared to those in the non-lesional aortic intimal tissues (*p<0.05; **p<0.01).

**Supplemental Figure II**

**miR155 expression is up-regulated in macrophages via TLR4 in inflammatory responses.**

A. qRT-PCR analysis of macrophage miR155 expression. Macrophages from WT, apoE-/-/LDLR-/—, and TLR4-/— mice were treated with mmLDL or LPS for 6 h. n=3 in each group, *p<0.01 vs DMEM, #p<0.01 vs WT with the same treatment. B. Macrophages of various genotypes were measured for expression levels of miR155, TNFα and IL-6 by qRT-PCR, after the treatment of LDL, acLDL, oxLDL or LPS. The correlation was analyzed.

**Supplemental Figure III**

**Manipulation of miR155 expression by lentiviral transduction.**

A. Lentiviral vector for miR155 overexpression. B. Lentiviral transduction of mouse macrophage at MOI of 30 increased miR155 expression by 30-40 folds.

**Supplemental Figure IV**

**Body weight and lipid parameters of the mice at the end point.** The 4-month-old DKO mice and apoE-/— were fed a Western-type diet for 12 week, and then sacrificed. The body weighs, the plasma total cholesterol and triglyceride levels, as well as FPLC lipoprotein profiles were measured.

**Supplemental Figure V**

**En face aorta atherosclerosis analysis of the mice.** At the time of sacrifice, mice heart were perfused with 1x PBS. The hearts and whole aortas were separated from the other tissues. Aortas were cut out from the heart below the tricuspidal valve and fixed in formalin at room temperature overnight and then kept in PBS before removing fatty tissue. The en face aortas were stained with Oil Red O and the plaque were analyzed by ImageJ.

**Supplemental Figure VI.**

Representative images of atherosclerotic lesions in apoE-/— or apoE/miR155 DKO (DKO) mouse proximal aorta. The lesions were stained using a Movat Pentachrom Stain Kit. Magnification: 60 X. Bar: 250 μm.

**Supplemental Figure VII.**

Representative images of hematoxylin stained atherosclerotic lesions in mouse proximal aorta before and after the lesions were dissected and removed. Magnification: 60 X. Bar: 250 μm.
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Supplemental Figure VII.
Representative images of hematoxylin stained atherosclerotic lesions in mouse proximal aorta before and after the lesions were dissected and removed. Magnification: 60 X. Scale bar: 250 µm.
Methods

Materials and reagents
Dulbecco’s Modified Eagle Medium (DMEM), RPMI 1640 and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). \(^{3} \text{H}\)-labeled cholesterol was from PerkinElmer (Waltham, MA). Acetylated LDL (ace-LDL) was from Biomedical Technologies (Stoughton, MA). All tissue culture plasticware was from Corning (Corning, NY). Pam3CSK4, Fsl-1, poly (I:C) and ODN1668 were all from InvivoGen (San Diego, CA). Lipopolysaccharide (LPS) was from Sigma-Aldrich (St. Louis, MO). Anti-SOCS-1 antibody was from Abcam (Cambridge, MA). Anti-SHIP-1 (V-19) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β–actin antibody and 25% glutaraldehyde were from Sigma-Aldrich. Anti-PECAM-1(CD31) and HRP-conjugated secondary antibodies were from Millipore (Bedford, MA). Antibodies against mouse ABCA1 and ABCG1 were purchased from Novus Biologicals (Littleton, CO). Primer for qPCR was from Integrated DNA Technologies (Coralville, IA). Primers for U6 and miR155 were from Qiagen (Valencia, CA). The human aorta samples were retrieved from forensic autopsy cases at Lexington hospital which were performed less than 8 hours after death. The human sample collection procedure was approved by Lexington Hospital under IRB #03-168. All atherosclerotic lesions were taken from either thoracic or abdominal aortas. Sections were fixed in histchoice for histologic examination and snap frozen in liquid nitrogen and stored at -80°C.

Mice
All mice were all purchased from The Jackson Laboratories (Bar Harbor, Maine) and housed in the University of South Carolina Animal Research Facility. Animal care procedures and experimental methods were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Carolina. miR155-/mice were crossbred with apoE- mice to generate miR155-/apoE- double knockout (DKO) mice. The 8-week-old female DKO mice and apoE- mice were fed a Western-type diet for 12 weeks, and then sacrificed for atherosclerosis lesion analysis. The western diet contains 21% anhydrous milkfat, 34% sucrose, and a total of 0.2% cholesterol (Diet 3 TD.88137; Harlan Laboratories, Indianapolis, IN).

Macrophage preparation and manipulation
Peritoneal macrophages were collected from mice which were injected with 3 ml 3% thioglycollate three days before, seeded in 6-well plates at 3 x 10^6 cells per well, and incubated with serum-free DMEM at 37 ºC overnight before further treatment. Treated macrophages were either lysed with Trizol reagent for RNA extraction and quantitative real-time PCR (qPCR), or lysed with RIPA buffer with 1% proteinase inhibitor and 1% phosphatase inhibitor cocktail for western blotting.

Lentiviral vector generation and lentiviral transduction
General procedures for lentivirus preparation and macrophage transduction were described previously. To generate a lentiviral vector for mouse and human miR155 overexpression, a 419-bp and 360-bp DNA fragment containing mouse or human pre-miR155 stem-loop were amplified by PCR from the LPS-stimulated C57BL/6 mouse macrophage cDNA and human THP-1 cell cDNA, respectively, and then sub-cloned into the bi-cistronic lentiviral vector PWPI.

Macrophage cholesterol efflux
Attached mouse macrophages in 24-well plates were cultured in DMEM for 24 h before cholesterol-loading with 100 µg/ml ace-LDL incorporated with \(^{3} \text{H}\)-cholesterol for 3 d. Then, efflux medium (either FBS-free DMEM or DMEM containing apoAI) was added to initiate cholesterol efflux for 24 h. \(^{3} \text{H}\) radioactivity in efflux medium and cells were counted using a Beckman LS6500 liquid scintillation counter (Beckman Coulter, Indianapolius, IN).

Quantitative Real-Time PCR
Total RNA was extracted using RNasy kit (Cat# 74104, Qiagen) and reverse transcribed into cDNA using Bio-Rad iScript cDNA Synthesis kit (Cat# 170-8891, Bio-Rad). qPCR measurement of target mRNA levels was performed on an Eppendorf Realplex2 Mastercycler (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions. MicroRNAs were extracted using miRNeasy Mini kit (Cat# 217004, Qiagen) and were reverse transcribed by miScript-II Reverse Transcription Kit (Cat# 218161, Qiagen). qPCR of microRNA was performed using miScript SYBR Green PCR Kits (Cat# 218073,
Qiagen). Mouse inflammatory gene expression was normalized with 18S RNA, while miR155 expression was normalized with U6, as endogenous controls, respectively. Following primers were used: Mouse 18s: 5’ CGCGGTTCTATTTGTGTTG 3’ (forward) and 5’ AGTGGCATCGTTATGGTC 3’ (reverse); mouse TNFα: 5’ CGTCAGCCATTCTATCTAT 3’ (forward) and 5’ CCGACTCCGCAAAGTCTAAG 3’ (reverse); mouse IL-6: 5’ AGTTGCTTCTTGGGACTGA 3’ (forward) and 5’ TCCACGATTTCAGAGAAG 3’ (reverse); mouse IL-1β: 5’ GCCCATCCTCGATGACTCAT 3’ (forward) and 5’ AGGCCACAGGTTTTTGTCG 3’ (reverse); mouse ABCA1: 5’ AACAGTTTGTGGCCCTTTTG 3’ (forward) and 5’ AGTTCCAGGCTGGGGTACTT 3’ (reverse); mouse ABCG1: 5’ GAACCACTCAGTGGAT 3’ (forward) and 5’ AGCCGTAGATGAGAAG 3’ (reverse). miR155 expression was detected according to the manufacturer’s instructions using the miScript PCR System (Qiagen, Valencia, CA) and miScript Primer Assays (Cat# MS00001701 for mouse miR155; Cat# MS00031486 for human miR155).

**Western blot**

Thirty micrograms of total protein was loaded onto 10% SDS-PAGE gels for electrophoresis. The protein was then transferred to nitrocellulose membranes (Amersham Biosciences, Pittsburgh, PA). Primary antibody and HRP-conjugated secondary antibody were used to detect target proteins. Signal was detected using an ECL kit (Amersham Biosciences).

**Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISA measurement of TNFα and IL-6 in macrophage conditioned medium or mouse plasma was performed as described previously. Briefly, mouse TNFα and IL-6 protein concentrations in macrophage conditioned medium and in mouse plasma were measured by ELISA using kits from eBioscience (Cat# 88-7324-88 and 88-7064-88, respectively, San Diego, CA) following the manufacturer’s instructions. The macrophage conditioned culture medium was diluted by 90-180 folds for the measurement. The 96-well microplates were read using a SpectraMax M5 microplate reader.

**Flow cytometry**

Flow cytometric analyses of leukocyte profile in mouse spleen were performed as described previously. Splenocytes were prepared by mechanical dissociation. Cells were stained with anti-CD11b PE mAb, anti-ly6C FITC mAb, anti-CD3 PE mAb, anti-CD4 FITC mAb, anti-CD8 FITC mAb, anti-CD19 FITC mAb (all from eBioscience) in staining buffer (PBS containing 2% FBS) for 30 min on ice in the dark. Samples were washed twice in staining buffer, analyzed by flow cytometry using a Cytomics FC 500 flow cytometer and CXP software version 2.2 (Beckman coulter, Brea CA). Data were collected for 10,000 live events per sample. We included all 10,000 live cells for the analysis to count the cell number for each cell type. For IL-17A and FoxP3 staining, splenocytes were firstly cultured in RPMI 1640 complete medium with 1 µg/ml Ionomycin (Sigma-Aldrich, St. Louis, MO, USA) and 50 ng/ml phorbol myristate acetate (PMA) (Sigma) in the presence of 2 µl of Golgi Stop (BD Biosciences, San Jose, CA, USA) for 10 h at 37°C. Cells were subsequently stained with anti-CD4 FITC and then permeabilized and fixed in Fixation/Permeabilization solution (eBioscience) at 4°C. Cells were washed with Perm/Wash™ buffer (eBioscience) and then stained with anti-IL-17A PE or anti-FoxP3 PE (eBioscience) for 30 min at 4°C. After washing, stained cells were analyzed as described above.

**Bone marrow transplantation**

Bone marrow transplantation (BMT) was performed as described previously. Briefly, recipient apoE−/− mice (12 weeks old, male or female) were lethally irradiated (900 rad). Bone marrow cells were harvested from apoE−/− or apoE−/−/miR-155−/− donor mice by flushing the femurs and tibias with phosphate buffered saline (PBS) supplemented with 2% FBS. The flushed bone marrow cells were resuspended in PBS, and 5 x 106 nucleated cells were injected retro-orbitally into irradiated mice within 6 h after irradiation. Four weeks after bone marrow transplantation, recipient mice were fed the western diet for 16 weeks.

**Plasma Lipid Analysis**

Plasma Cholesterol and triglyceride were determined by enzymatic colorimetric assays using Cholesterol Reagent and Triglycerides GPO reagent kits (Raichem, San Diego, CA, Catlog # R80035 and 339-10, respectively). Plasma lipoprotein profiles were determined by fast-performance liquid
chromatography (FPLC) using a Superose 6 10/300 GL column on an AKTA purifier (GE Healthcare).

**Atherosclerosis Analysis**

At termination, mouse heart was perfused with 1x PBS. The Aortic root was embedded in OCT medium and frozen in -20 °C immediately after being cut away from aorta. The aorta was dissected and fixed in 10% neutral buffered formalin at room temperature overnight and then kept in PBS before removing fatty tissue. For analysis of atherosclerosis in the aorta root, 10-µm serial sections were collected. The lesion area of the frozen sections were detected by H&E staining (hematoxylin, GeneTex, Inc. ready-to-use, cat#GTX73341; eosin, sigma-aldrich, eorsin B, cat#61006-10G), the lipid-stained lesions were detected by Oil Red O staining and quantified by Image-Pro Plus 6.0. Rat anti-mouse moma2 (Abcam) was used for immunostaining in sections from aortic sinuses of mice for macrophage analysis, and biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; Cat# BA-1000) as the secondary antibody. Immunoreactivity was visualized using the Vectastain ABC kit (Vector Laboratories, Cat#PK6100), and signal was enhanced by Peroxidase Enhancer (GeneTex, Irvine, CA; GTX82979), and finally reacted with the substrate from AEC Chromogen/FRP substrate kit (GeneTex, GTX82977). The en face aortas were stained with Oil Red O and the plaque areas were analyzed by ImageJ. Proper controls were used as described previously. For Movat’s Pentachrome staining of the lesions, the 10 micron thick aortic root sections were stained by Movat’s Pentachrome using a Movat Pentachrome Stain Kit from Scy Tek Laboratory (Cat# MPS-2, Logan, UT) following the manufacturer’s instructions and analyzed by Image-Pro Plus 6.0.

**Laser capture microdissection (LCM)**

LCM analysis was performed generally following the protocols published by Fisher and colleagues. Briefly, 6-µm-thick sections were cut from the frozen aortic root on a cryostat and placed on PEN membrane-covered, nuclease and human nucleic acid-free slides. The sections were quickly stained with hematoxylin and the entire atherosclerotic lesions were dissected on a Zeiss PALM CombiSystem (Zeiss, Thornwood, NY). Lesions from 8 sections of each mouse were pooled to form one sample and total RNAs were extracted for qPCR analysis of target genes.

**Statistical analysis**

Data are presented as mean ± the standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism software 5.0 (GraphPad Software, Inc. La Jolla, CA). Differences between two groups were determined by Student’s t-tests, given the distribution of the data was normal. For correlation analysis, linear regression was used. A p<0.05 was considered as statistically significant.

**References**